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TITLE: Inflammation as a Driver of Clonal Evolution in Myeloproliferative Neoplasm

PRINCIPAL INVESTIGATOR: Angela Fleischman

CONTRACTING ORGANIZATION: University of California, Irvine
Irvine, CA 92617

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Angela Fleischman, Sarah Morse, Stefan Brooks, Hew Yeng Lai, Brianna Craver, Nitya Mehrotra

E-Mail: agf@uci.edu

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14. ABSTRACT
Tumor Necrosis Factor-alpha (TNF) is elevated in myeloproliferative neoplasm (MPN) and plays a key role in expansion of the *JAK2*^{V617F} neoplastic clone. We have found that MPN monocytes produce excessive amounts of TNF in response to TLR ligation due to a defect in the negative regulatory feedback loop which normally serves to dampen TNF production. We have localized this defect to a blunted response to the anti-inflammatory cytokine IL-10. MPN monocytes are less responsive to the anti-inflammatory actions of IL-10 at low concentrations but these effects can be restored by increasing the concentration of IL-10. Together, these data suggest that administration of IL-10 may reduce excessive inflammatory cytokine production in MPN.

15. SUBJECT TERMS
Myeloproliferative neoplasm, JAK2V617F, IL-10, TNF, inflammation

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Table of Contents

Page

1. Introduction

Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Excessive production of inflammatory cytokines such as TNF is a characteristic feature of MPN. Excessive inflammation is not only responsible for the debilitating constitutional symptoms associated with this disease but also plays a central role in MPN disease initiation and progression. However, the mechanism causing excessive inflammation in MPN is not known. As a potential consequence of this gap in knowledge, MPN patients have no pharmacologic therapeutic options that alter the natural history of their disease. In order to accurately target harmful inflammation in MPN while preserving critical anti-inflammatory pathways, we must first define the mechanism driving excessive inflammation in MPN. The overall objectives of this project are to define the mechanism driving excessive TNF production in MPN and to identify agents that have the ability to dampen TNF production in MPN for further development as therapeutics.

2. Keywords

Myeloproliferative neoplasm, JAK2V617F, IL-10, inflammation, TNF, TLR signaling

3. Accomplishments

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

- **What were the major goals of the project?**
  - *List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project identify these dates and show actual completion dates or the percentage of completion.*

The major goals of this project are to define the mechanism causing excessive TNF production in MPN patients, to identify the role of JAK2V617F in abnormal TLR signaling, and to restore the TLR negative feedback loop as a therapeutic approach in MPN.

- **What was accomplished under these goals?**
  - *For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

We have previously observed that MPN monocytes exhibit a dampened response to IL-10. We hypothesized that this may be explained in part by reduced cell surface expression of IL-10R in MPN patients compared to normal controls. To this end, we isolated peripheral blood mononuclear cells from MPN patients and normal controls. We then used flow cytometry to analyze IL-10R cell surface expression by mean fluorescence intensity (MFI) on CD14+ cells (monocytes) and found that IL-10R expression is not significantly different between MPN and normal samples (Figure 1).

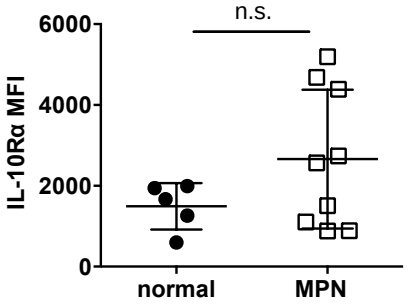

Figure 1. IL-10R expression. MPN (n=9) and normal (n=5) PBMCs were stained for CD14 and IL-10R. IL-10R expression was not significantly different between MPN and normal monocytes.

As the dampened response to IL-10 observed in MPN cells could not be explained by reduced expression of IL-10R, we next assessed whether MPN monocytes displayed defects in the IL-10 signaling cascade. We stimulated MPN and normal monocytes with IL-10 then used phosphoflow to analyze phosphorylation of STAT3, a key component in the IL-10 signal transduction pathway. We determined that phospho-STAT3 is markedly reduced in MPN monocytes as compared to normal controls upon IL-10 stimulation (Figure 2). This indicates that MPN cells are less able to activate STAT3 in response to IL-10. Our results also demonstrated that MPN monocytes increase phosphorylation of STAT3 proportionate to IL-10 concentration, providing support for the therapeutic use of exogenous administration of high doses of IL-10 to reduce inflammation in MPN patients.

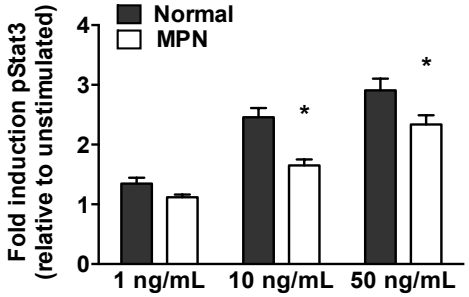

Figure 2. Defective IL-10 signaling in MPN patients. MPN and normal monocytes were stimulated for 15 minutes with IL-10 at the concentrations shown prior to fixation and permeabilization. Cells were stained for pStat3 and analyzed via flow cytometry. pStat3 induction was significantly reduced in MPN monocytes (n=19) compared to normal controls (n=18).

Phosphorylated STAT3 translocates to the nucleus where it induces expression of negative regulators of inflammation, including SOCS3. We investigated whether the reduced STAT3

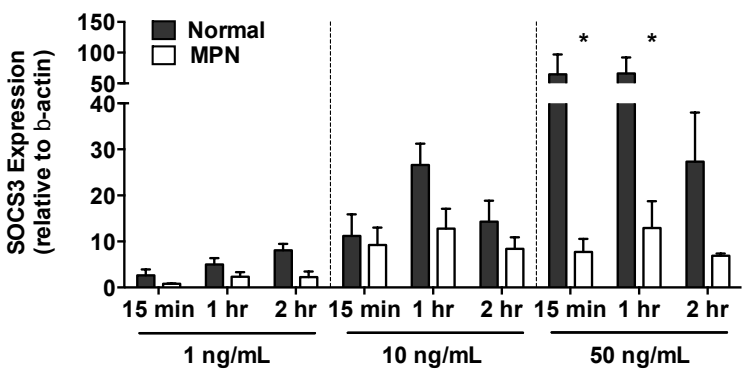

Figure 3. Defective IL-10 signaling in MPN patients. MPN and normal monocytes were stimulated with IL-10 for 15 min, 1h, or 2h at the concentrations shown then lysed in trizol. RNA was isolated and reverse-transcribed to cDNA. SOCS3 mRNA was quantified by qPCR and normalized to b-actin IL-10 induced less SOCS3 expression in MPN monocytes (n=3) compared to normal monocytes (n=3).

phosphorylation we observed in MPN monocytes in response to IL-10 stimulation corresponded to a reduced ability to transcribe SOCS3. This revealed a significant reduction in the ability of MPN monocytes to induce SOCS3 in response to IL-10 at 15 minutes and 1 hour compared to normal controls (Figure 3).

We have demonstrated that MPN patients have a dampened response to IL-10 due to impairments in the IL-10 signaling cascade. However, the MPN patient samples examined thus far have contained populations of both wild-type and JAK2V617F-mutated cells. We sought to investigate the role of JAK2V617F in TNF overproduction. To this end, we stimulated MPN monocytes with LPS and added brefeldin-A to block Golgi trafficking and trap TNF within the cells. Brefeldin-A was added either alongside LPS (0h) or 6 hours after LPS stimulation. Cells were incubated for 4h followed by sorting for TNF+ and TNF- fractions. DNA was isolated from both cell fractions and JAK2V617F allele burden was then determined via qPCR. We found that JAK2V617F allele burden was similar between TNF+ and TNF- populations, indicating that the prolonged TLR response observed in MPN patients is not specific to the JAK2V617F-mutated cells (Table 1).

Table 1: Allele burden (% Jak2^{V617F})

	0 h		6 h	
Patient	**TNFα-**	**TNFα+**	**TNFα-**	**TNFα+**
192	92.21	57.52	85.58	82.65
228	63.66	57.92	90.01	78.10
232	34.88	42.66	53.13	51.90
252	94.84	90.50	66.20	63.45
255	20.95	76.86	75.24	80.23

To further determine the effect of JAK2V617F on the TLR signaling cascade, we plan to ectopically express JAK2V617F, Jak2WT, or MSCV-IRES-GFP (MIG) empty vector by retroviral infection in the macrophage cell line RAW264.7. We will measure TNF and IL-10 at 0, 3, 6, 9, 18, and 24 hours after LPS stimulation by intracellular flow cytometry, ELISA, and qRT-PCR. To determine whether JAK2V617F directly affects the intensity or duration of activation of key downstream signaling molecules, we will measure phosphorylation of ERK1/2 and MAPK after LPS stimulation and phosphorylation of STAT3 after IL-10 stimulation.

Our findings thus far have indicated that JAK2V617F-mutated cells are not directly responsible for excessive TNF production in MPN patients. The transplantation-transduction mouse model of JAK2V617F recapitulate the excessive TNF production found in MPN patients, although the mechanism by which JAK2V617F achieves this is unknown.

We next aim to determine the effect of JAK2V617F cells on TLR signaling of bystander normal monocytes. In our transduction-transplantation model, mice harbor both mutant and wild-type cells, with the JAK2V617F cells marked by GFP. We will isolate GFP+ and GFP- mouse monocytes from JAK2V617F and MIG empty vector transplanted mice and stimulate with LPS for 24 hours. The amount of TNF in the supernatant will be quantified by ELISA.

Restoring the TLR negative feedback loop would reduce inflammation in MPN and we hypothesized that this would also reduce the neoplastic clonal burden. We will screen candidate compounds for the ability to reduce TNF production by LPS-stimulated MPN monocytes. Compounds that we identify will then be tested for their ability to attenuate disease in a mouse

MPN model. Preliminarily, we have assessed the abilities of the compounds listed in Table 2 to reduce TNF production upon LPS stimulation of the RAW264.7 macrophage cell line.

Table 2: Agents with predicted ability to reduce TNF production in MPN monocytes

AGENT	MECHANISM OF ACTION	RATIONALE FOR SELECTION
Ruxolitinib	JAK1/2 inhibitor	Decreases TNF in clinical trials in MPN patients
Trametinib	ERK1/2 inhibitor	We have recently found ERK to be activated in MPN mouse models and MPN patients
N-acetyl cysteine	Antioxidant	Decreases TLR signaling in animal model of LPS induced inflammation
Curcumin	Inhibits NFKB	Decreases chronic inflammation and decreases LPS induced TNF production

We have identified trametinib as a potent inhibitor of LPS-induced TNF production in the RAW264.7 cell line at all time points tested (Figure 8). Conversely, ruxolitinib treatment did not alter TNF production after TLR activation.

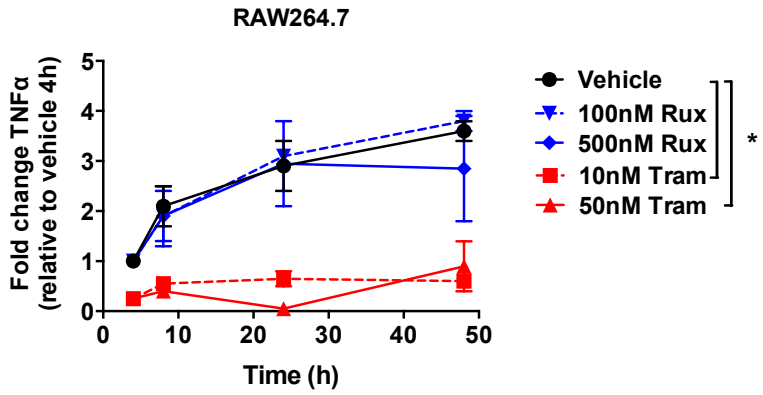

Figure 6. Reduced TNFα production with trametinib treatment. The mouse macrophage cell line RAW264.7 was pre-treated with ruxolitinib or trametinib for 1 h at the indicated concentrations and stimulated with LPS for 4, 8, 24, or 48h. Supernatants were harvested and TNFα was measured by ELISA. Trametinib, but not ruxolitinib, reduced TNFα production at all time points.

We next examined the ability of trametinib and ruxolitinib to reduce LPS-induced TNF in MPN patients and normal controls (Figure 7). Peripheral blood mononuclear cells (PBMCs) were isolated from MPN patients and normal controls. Cells were treated with drug alone or in the presence of 10 ng/ml IL-10 and stimulated with LPS. TNF was quantified by ELISA. In both normal and MPN PBMCs, trametinib treatment inhibited TNF production upon TLR activation, while ruxolitinib did not. Additionally, treatment with ruxolitinib, a JAK1/2 inhibitor, blocked the ability of IL-10 to reduce TNF production. As IL-10R induces phosphorylation of JAK1 upon activation, this is unsurprising but represents a major drawback to ruxolitinib therapy. Our findings thus far suggest that the ideal therapeutic compound would reduce TNF and increase IL-10 production. While our preliminary results indicate that trametinib is a promising compound for TNF reduction, further work is needed to determine whether trametinib alters IL-10 signaling or expression. We also plan to investigate the effects of N-acetyl cysteine and curcumin treatment on TNF and IL-10 production in normal and MPN PBMCs.

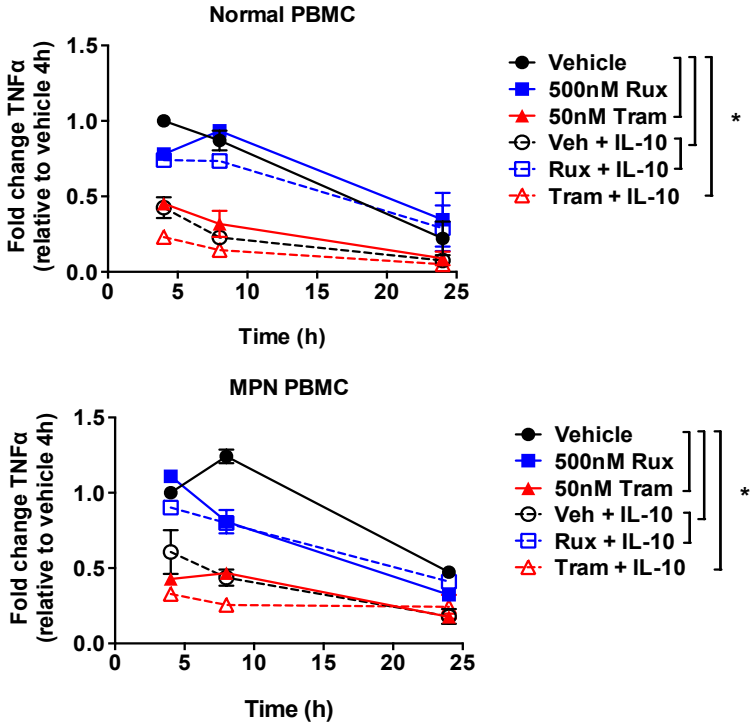

Figure 7. Reduced TNF production with trametinib treatment. Normal and MPN PBMCs were pre-treated with ruxolitinib or trametinib for 1h at the indicated concentrations then stimulated with LPS for 4, 8, or 24h. Supernatants were harvested and TNF was measured by ELISA. Trametinib, but not ruxolitinib, significantly reduced TNF production at all time points. Ruxolitinib treatment blocked the ability of IL-10 to reduce TNF production.

- **What opportunities for training and professional development has the project provided?**

  This project has allowed me to participate in UC Irvine's Institute of Immunology seminar series and journal clubs. These activities and interactions with immunologists have allowed me to better understand the intricacies of the Toll like receptor signaling pathway.

  - *If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."*

- *Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.*

- **How were the results disseminated to communities of interest?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.*

    I have presented this work in oral format at the La Jolla Immunology Conference. I have also spoken about my work to a lay audience on three occasions for the Leukemia Lymphoma Society.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - *If this is the final report, state "Nothing to Report."*
  - *Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.*

    We will continue our current pace on experiments. In the next reporting period we expect to evaluate the effect of IL-10 treatment in a mouse MPN model. We also anticipate submission of a manuscript on our work accomplished thus far in the next 2 months.

4. Impact

Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

- **What was the impact on the development of the principal discipline(s) of the project?**

  Our finding that persistent TLR signaling is seen in MPN and that this could potentially be a predisposing factor to acquire MPN has significant relevance for the development of preventative strategies in MPN.

- *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
- *Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

- **What was the impact on other disciplines?**

  Nothing to Report

  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.*
- **What was the impact on technology transfer?**

  Nothing to Report

  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:*
    - *transfer of results to entities in government or industry;*
    - *instances where the research has led to the initiation of a start-up company; or*
    - *adoption of new practices.*
- **What was the impact on society beyond science and technology?**

  We are developing a low inflammatory diet intervention in MPN. This may lead to changes in MPN patients in terms of heightening their awareness of healthy eating practices.

  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:*
    - *improving public knowledge, attitudes, skills, and abilities;*
    - *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
    - *improving social, economic, civic, or environmental conditions.*

5. Changes/Problems

Nothing to Report

The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

- **Changes in approach and reasons for change**

  No Changes

  - *Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.*
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - *Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

  The transduction-transplantation mouse model has been delayed due to issues with the quality of JAK2V617F retrovirus. New materials have been purchased and methods have been optimized to improve the titer of the retrovirus.

  We anticipated that acquiring a sufficient quantity of MPN patient samples for this work would take some time. We have utilized cell lines where possible. We are continuing to recruit patients for this study. We are also working to ectopically express JAK2WT, JAK2V617F, and MIG empty vector in the RAW264.7 cell line. We plan to determine whether the JAK2V617F-mutated cells exhibit an exaggerated TLR response by ELISA and intracellular staining. If so, we will utilize this cell line to test candidate compounds for the ability to reduce TNF and increase IL-10.

- **Changes that had a significant impact on expenditures**

  None

  - *Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.*
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  None

- *Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*

  Nothing to report.

6. Products

List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.
  - **Journal publications.** *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

    Nothing to report.

  - **Books or other non-periodical, one-time publications.** *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

    Nothing to report.

  - **Other publications, conference papers, and presentations.** *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

    Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research

activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

  Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

  Nothing to Report

7. Participants & Other Collaborating Organizations

- **What individuals have worked on the project?**
  - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals*

approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

***Example**:*

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Name:	Angela Fleischman
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-3701-6079
Nearest person month worked:	2
Contribution to Project:	Dr. Fleischman has designed the experiments, performed experiments, and analyzed data.
Funding Support:	DoD Career Dev Award, V Foundation Scholar Award, Clinical Revenue, FTE from University of California

Name:	Sarah Morse
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	none
Nearest person month worked:	3
Contribution to Project:	Ms. Morse has processed patient blood samples, performed qPCR, and analyzed data.

Funding Support:	DoD Career Development Award, V Foundation Scholar Award, Start Up package funds

Name:	Hew Yeng Lai
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	Does not have one
Nearest person month worked:	2
Contribution to Project:	Ms. Lai has processed patient blood samples, performed ELISA and intracellular staining experiments, and analyzed data.
Funding Support:	UC Irvine PhD program (covered by program not PI for first year of PhD)

Name:	Stefan Brooks
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	0000-0003-1670-1939
Nearest person month worked:	1
Contribution to Project:	Mr. Brooks has performed qPCR to assess SOCS3 expression.
Funding Support:	NCI T32 Training Grant

Name:	Brianna Craver
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	none
Nearest person month worked:	2
Contribution to Project:	Ms. Craver has performed ELISA to assess candidate compounds for their ability to reduce TNF.

Funding Support:	UC Irvine PhD program, Start Up Package

Name:	Nitya Mehrotra
Project Role:	Student Volunteer
Researcher Identifier (e.g. ORCID ID):	none
Nearest person month worked:	1
Contribution to Project:	Ms. Mehrotra has screened candidate compounds for TNF reduction via intracellular staining and ELISA.
Funding Support:	None

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  V Foundation Scholar Plus Award – additional $100,000 for 1/1/17-12/31/17

  MPN Research Foundation Challenge Grant - $100,000 per year x 2 years 10/1/17-9/30/19

  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.*
- **What other organizations were involved as partners?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe partner organizations - academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) - that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.*

Nothing to report.

8. Special Reporting Requirements

- **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.*
- **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

Nothing to report.

9. Appendices

*Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***

None